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Inhibition of tumor metastasis of Lewis lung carcinoma in C57BL/6 mice by intrapleural administration of *Lactobacillus casei*

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Summary. The antimetastatic effect of *Lactobacillus casei* YIT9018 (LC 9018) against Lewis lung carcinoma (3LL) in C57BL/6 mice was determined. Intrapleural (i. pl.) administration of LC 9018 was effective in inhibiting pulmonary metastasis after s. c. inoculation of 3LL tumors into C57BL/6 mice. The combination of i. pl. and intralesional or i. v. injections of LC 9018 also markedly inhibited pulmonary metastasis in 3LL-bearing mice. The i. pl. administration of LC 9018 into mice induced an increase in the number of thoracic exudate cells (TEC) and the cell population in the TEC was mainly polymorphonuclear leukocytes in the early stage, while macrophages were dominant in the late stage. In addition, in vitro cytolytic activity against 3LL cells and natural killer cell activity of TEC were augmented by the i. pl. administration of LC 9018. Furthermore , i. pl. administration of LC 9018 into the mice rendered their lung macrophages tumoricidal for 3LL cells in vitro. These results show that TEC induced by i. pl. administration of LC 9018 played a key role in the inhibition of metastasis in 3LL-bearing mice.

Introduction

Lewis lung carcinoma (3LL) has been widely used as one of the hematogenous metastasis models and marked metastasis occurs in the lungs of C57BL/6 mice after s. c. or i. d. implantation of the tumor cells [2, 3]. The metastasis takes place in various steps and its detailed mechanisms have not yet been clarified. Inhibition of metastasis is one of the most important problems in cancer therapy. Many investigators have studied cancer immunotherapy using microorganisms such as Bacillus Calmette-Guérin cell wall skeleton (BCG-CWS), *Corynebacterium parvum* (*C. parvum*), and the cell wall skeleton of *Nocardia rubra* (N-CWS) in experimental animals and clinical trials [1, 9, 12]. The route of administration of immunoadjuvants is one of the most crucial problems concerning the immunotherapy of cancer. In animal experiments, i. p., s. c., i. v. or intralesional (i.l.) injection has been widely used to investigate the effect of immunoadjuvants. Intraperitoneal, s. c., or i.l. injection provides an opportunity to study immunotherapy by the reaction at the local site in the host, and i. v. injection gives an opportunity to study the systemic reactions of the host. On the other hand, intrapleural (i. pl.) injection of immunoadjuvants has been little used for cancer therapy in experimental animals, while in clincal trials, it has been reported that i. pl. injection of BCG-CWS, N-CWS, or OK432 is effective for lung cancer patients with malignant pleurisy [14–18].

The heat-killed cells of *Lactobacillus casei* YIT9018 (LC 9018), a gram-positive and nonpathogeneic organism, have been reported to exhibit potent antitumor activity in experimental mouse tumor systems [7]. It has also been reported that LC 9018 has antimetastatic [10, 11] and antilisterial effects [13] in mice and guinea pigs. The effect of LC 9018 was considered to be host-mediated because of the absence of direct cytotoxicity for tumor cells in vitro [7]. LC 9018 has also been reported to be able to augment the macrophage function in vitro or in vivo, as judged by the ability to produce oxygen radicals [4], or by an increase in phagocytic activity [8].

In the present study, by using experimental transplantable tumor models in syngeneic mice we investigated the antitumor activity of i. pl. administration of LC 9018, especially its antimetastatic effect. The cytolytic and natural killer (NK) activities of thoracic exudate cells (TEC) and the cytolytic activity of lung macrophages induced by i. pl. administration of LC 9018 in C57BL/6 mice were also determined.

Materials and methods

Animals. Specific pathogen-free male C57BL/6J mice, 7–10 weeks old, were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. They were kept in plastic cages and given food and water freely.

Tumors. The 3LL was maintained by serial s. c. transplantation in C57BL/6 mice. After aseptic removal, 3LL tumors were minced in Hanks' balanced salt solution (HBSS) and the cell suspension filtered through a stainless steel mesh. Tumor cells were collected by centrifugation and resuspended in HBSS. The tumor cells were counted using a hemocytometer and adjusted to the desired concentration. YAC-1 lymphoma cells, as the target cells of NK cells, were maintained in vitro in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS).

Preparation of LC 9018 and c. parvum. The LC 9018 was cultured for 24 h at 37° C in Rogosa's medium [10]. After cultivation, the cells were collected by centrifugation,

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with ion-exchanged water, killed by heating for 30 min at 100° C and lyophilized. These lyophilized cells were reconstituted with sterile saline before use. The *C. parvum* was purchased from the Institute Merieux, Lyon, France, and used as a reference adjuvant.

Population of TEC. The LC 9018 (250 μg/mouse) was injected i.pl. into mice (10 mice/group) on day 0, and TEC were collected on days 1, 3, 5, 7, 10, and 14 by washing the thoracic cavity of the mice with 2.5 ml of HBSS. Then, the TEC suspension was centrifuged and resuspended in HBSS. The total number of TEC was counted using a hemocytometer. A cytocentrifuge preparation was made with the TEC suspension by staining with May-Grünwald's and Giemsa's solutions and examined microscopically. A differential cell count was performed by counting more than 100 cells and the percentage of each cell type calculated.

Antimetastatic effect of LC 9018. The 3LL cells (5×10^5) were inoculated into the left groin of mice (7 mice/group) on day 0. LC 9018 was injected i.pl. (Table 1), i.pl. and/or i.l. (Table 2), and i.pl. and/or i.v. (Table 3) on days 7, 10, 13, 16, and 19 after tumor cell inoculation. The mice were killed on day 28 and the lungs weighed and examined for pulmonary nodules. The number of metastatic foci was determined by counting the surface colonies.

Preparation of thoracic macrophages. The LC 9018 (250 μg/mouse) was injected i.pl. into mice (10 mice/group) on day 0, and TEC were collected by washing the thoracic cavity of the mice with 2.5 ml of HBSS. Then, the TEC suspension was centrifuged and resuspended in HBSS. The TEC suspension was placed in Percoll solution (density 1.070, Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged (1,500 xg, 20 min). The macrophage-enriched population was determined microscopically by staining with May-Grünwald's and Giemsa's solutions. The macrophage-enriched fraction (>97%) was aspirated using a Pasteur pipette and washed twice with HBSS. The macrophage-enriched fraction was then suspended in RPMI 1640 medium supplemented with 10% FBS. The cells were counted using a hemocytometer and adjusted to the desired concentration.

Cytolytic activity of thoracic macrophages. The LC 9018 (250 μg/mouse) was injected i.pl. into mice (10 mice/group) on day 0, and TEC were collected by washing the thoracic cavity of the mice with 2.5 ml of HBSS on days 1, 3, 5, 7, 10, and 14. Thoracic macrophages were harvested as described above and a thoracic macrophage-mediated cytotoxicity assay was performed using the ^{51}Cr release method. In brief, 3LL cells (1×10^6) as target cells were labeled by incubation with 100 μCi of $Na_2{}^{51}CrO_4$ (new England Nuclear Corp., Boston, Mass., USA) at 37° C for 1 h in a shaking water bath. After incubation, the cells were washed three times with HBSS to remove unbound radiolabel and resuspended in RPMI 1640 medium containing 10% FBS. The target cells were placed in a 96-well microtiter plate (Nunc, Roskilde, Denmark) and effector cells in a volume of 100 μl were added in triplicate at the desired concentration. The macrophage-target cell cultures were centrifuged for 5 min at 400 xg and then incubated for 18–20 h at 37° C in a humidified atmosphere containing 5% CO_2. After incubation, the plates were centrifuged for 7 min at 500 xg. The percentage specific release of ^{51}Cr was determined by removing 0.1 ml supernatant from each well and counting the activity in a gamma-scintillation counter (400 CGD, Packard Instrument Co., Inc., Downers Grove, Ill, USA). The percent cytotoxicity mediated by thoracic macrophages was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}} \times 100$$

NK cell activity. The LC 9018 (250 μg/mouse) was injected i.pl. into mice (10 mice/group) on day 0, and TEC were collected on days 1, 3, 5, and 7. The TEC suspension was incubated in a plastic dish at 37° C for 90 min, twice, to deplete plastic adherent cells. The plastic nonadherent cells were then used as the source of NK cells. NK cell activity was determined by the ^{51}Cr release method [6]. In brief, target cells in HBSS were labeled for 1 h at 37° C with 100 μCi of $Na_2{}^{51}CrO_4$ and the cells were washed three times with HBSS to remove unbound radiolabel. The cytotoxicity of effector cells for target cells was determined as described above for the assay of cytolytic activity of thoracic macrophages.

Cytolytic activity of lung macrophages. The LC 9018 (250 μg/mouse) was injected i.pl. into mice (10 mice/group) on day 0, and lung macrophages were collected on days 1, 3, 5, and 7. The lungs were removed aseptically and minced with HBSS. The lung suspension was incubated with 0.05% collagenase (Yakult Honsha Co. Ltd., Tokyo, Japan) at 37° C for 60 min, then centrifuged and resuspended in RPMI 1640 medium containing 10% FBS. The lung macrophages were collected by the Percoll gradient method as described above for the preparation of thoracic macrophages. The cytolytic activity of the lung macrophages was determined by the ^{51}Cr release method as described above for the cytolytic activity of thoracic macrophages.

Statistical analysis. Statistical analysis was performed using Student's *t*-test.

Results

Effect of i.pl. administration of LC 9018 on the number of TEC

We first determined the effect of i.pl. administration of LC 9018 on the induction of TEC in mice. The LC 9018 (250 μg/mouse) was injected i.pl. into mice on day 0, and the TEC were collected on days 1, 3, 5, 7, 10, and 14. Differential cell counts were performed microscopically on cytocentrifuge preparations of the TEC. The total number of TEC was increased on day 1 and then decreased gradually after the administration of LC 9018 (Fig. 1). In the population of TEC, the ratio of polymorphonuclear leukocytes (PMNs) was increased for 3 days, especially on day 1, and after that macrophages were dominant from day 3 to 14.

Antimetastatic effect of LC 9018 on 3LL

We determined the effect of i.pl. injection of LC 9018 on pulmonary metastasis in 3LL-bearing mice. The 3LL cells were inoculated s.c. into mice, and then LC 9018 was giv-

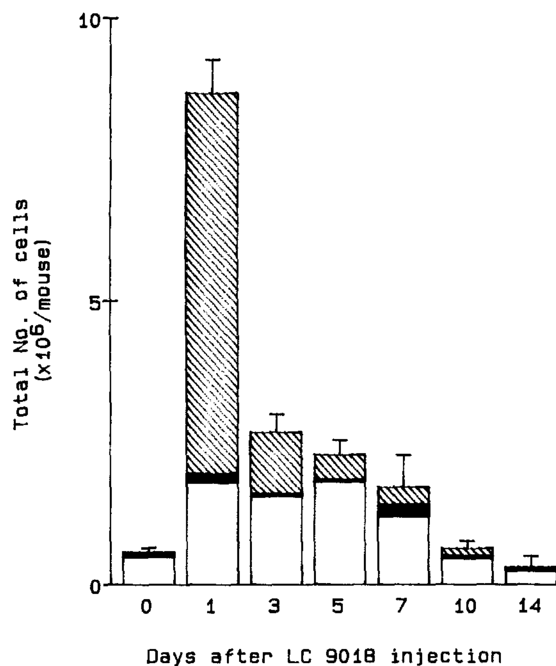

Fig. 1. Effect of i.pl. administration of LC 9018 on the number of TEC. *Lactobacillus casei* YIT9018 (LC 9018) (250 µg/mouse) was injected intrapleurally (i.pl.) into C57BL/6 mice (10 mice/group) on day 0. Thoracic exudate cells (TEC) were collected on days 1, 3, 5, 7, 10, and 14. Differential cell counts were performed microscopically with a cytocentrifuge preparation of the TEC. (▧ :PMNs, □ :macrophages, ■ :lymphocytes). *Bars:* SD of the total number of cells

en i.pl. at various doses on days 7, 10, 13, 16, and 19 after tumor cell inoculation. Intrapleural injection of LC 9018 inhibited pulmonary metastasis as effectively as *C. parvum* compared with the control group (Table 1). The combination of i.pl. and i.l. administration of LC 9018 was effective in inhibiting pulmonary metastasis (Table 2), and the combination of i.pl. and i.v. administration significantly inhibited lung metastasis (Table 3).

In vitro cytolytic activity of thoracic macrophages and NK activity of TEC

To determine the effect of LC 9018 on these activities, the cytolytic activity of thoracic macrophages against 3LL tumor was studied. The LC 9018 was injected i.pl. and thoracic macrophages were collected on days 1, 3, 5, 7, 10, and 14. The cytolytic activity of thoracic macrophages from LC 9018-treated mice was significantly higher than that of the control thoracic macrophages (Table 4). The activity was increased 3 days after the i.pl. injection of LC 9018 and the activity was maintained for more than 14 days. Furthermore, NK activity of TEC induced by i.pl. administration of LC 9018 was increased on day 3 and maintained for 7 days after LC 9018 administration (Table 5). These results indicate that i.pl. injection of LC 9018 was able to induce cells cytolytic for 3LL tumor cells in the thoracic cavity.

In vitro cytolytic activity of lung macrophages

Lung macrophages play an important role in preventing lung metastasis. We determined the cytolytic activity of

Table 1. Antimetastatic effect of i.pl. administration of LC 9018 on 3LL-bearing mice

Group[a]	Adjuvant	Dose[b]	No. of pulmonary metastases[c] (median)	Weight of lungs[c] (mg, mean ± SD)
1	–	Saline × 5	42, 40, 37, 36, 34, 31, 27 (36)	265 ± 40
2	LC 9018	250 µg × 5	23, 15, 12, 10, 9, 4, 3 (10)**	218 ± 26
3	LC 9018	100 µg × 5	21, 21, 18, 18, 11, 7, 6 (18)*	225 ± 16
4	LC 9018	50 µg × 5	38, 38, 37, 31, 29, 28, 11 (31)	248 ± 35
5	*C. parvum*	250 µg × 5	25, 18, 14, 10, 8, 6, 6 (10)**	228 ± 24

[a] Lewis lung carcinoma (3LL) cells (5 × 10^5/mouse) were inoculated s.c. into C57BL/6 mice (7 mice/group) on day 0
[b] LC 9018 or *Corynebacterium parvum* (*C. parvum*) was injected i.pl. on days 7, 10, 13, 16, and 19
[c] The number of pulmonary metastases and the weight of the lungs were determined on day 28 after tumor inoculation
Statistical significance of difference from Group-1: * $P < 0.01$, ** $P < 0.001$

Table 2. Antimetastatic effect of combined i.l. and i.pl. administration of LC 9018

Group[a]	Administration of LC 9018[b]		No. of pulmonary metastases[c] (median)	Weight of lungs[c] (mg, mean ± SD)
	i.l.	i.pl.		
1	–	–	62, 59, 45, 44, 42, 37, 33 (44)	326±52
2	–	+	24, 21, 20, 19, 16, 15, 15 (19)**	240±36
3	+	–	20, 19, 18, 16, 14, 12, 10 (16)***	217±30*
4	+	+	14, 12, 9, 8, 7, 5, 4 (8)***	202±21**

[a] 3LL cells (5 × 10^5/mouse) were inoculated s.c. into C57BL/6 mice (7 mice/group) on day 0
[b] LC 9018 (250 µg/mouse) was injected i.l. and/or i.pl. on days 7, 10, 13, 16, and 19
[c] The number of pulmonary metastases and the weight of the lungs were determined on day 28 after tumor inoculation
Statistical significance of difference from Group-1: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3. Antimetastatic effect of combined i.v. and i.pl. administration of LC 9018

Group[a]	Administration of LC 9018[b]		No. of pulmonary metastases[c] (median)	Weight of lungs[c] (mg, mean ± SD)
	i.v.	i.pl.		
1	–	–	45, 42, 39, 36, 34, 32, 29 (36)	253 ± 11
2	–	+	15, 14, 10, 10, 2, 1, 0 (10)**	242 ± 22*
3	+	–	29, 26, 15, 7, 6, 6, 4 (7)*	228 ± 16
4	+	+	5, 3, 2, 1, 0, 0, 0 (1)**	191 ± 26*

[a] 3LL cells (5×10^5/mouse) were inoculated s.c. into C57BL/6 mice (7 mice/group) on day 0
[b] LC 9018 (250 μg/mouse) was injected i.v. and/or i.pl. on days 7, 10, 13, 16, and 19
[c] The number of pulmonary metastases and the weight of the lungs were determined on day 28 after tumor inoculation
Statistical significance of difference from Group-1: * $P < 0.01$, ** $P < 0.001$

Table 4. In vitro cytolytic activity of thoracic macrophages from mice injected with LC 9018 i.pl. against 3LL

Days after LC 9018 injection[a]	^{51}Cr release	
	10 : 1[b]	5 : 1
0 (control)	4.0 ± 5.0[c]	−2.2 ± 3.8
1	3.9 ± 1.7	2.0 ± 1.0
3	41.5 ± 11.0	32.9 ± 5.2
5	49.3 ± 11.0	34.7 ± 10.3
7	39.0 ± 14.0	32.9 ± 10.1
10	28.5 ± 7.9	24.6 ± 6.0
14	26.0 ± 8.0	21.8 ± 8.6

[a] LC 9018 (250 μg/mouse) was injected i.pl. into C57BL/6 mice (10 mice/group) on day 0
[b] Effector/target ratio
[c] Mean ± SD of triplicate cultures

Table 5. Natural killer activity of TEC from mice injected with LC 9018 i.pl.

Days after LC 9018 injection[a]	Cytotoxicity[b]	
	50 : 1[c]	25 : 1
0 (control)	3.5 ± 2.0[d]	−1.0 ± 1.0
1	2.3 ± 1.6	1.9 ± 3.9
3	45.0 ± 6.6	30.0 ± 5.9
5	37.0 ± 2.6	28.9 ± 3.5
7	46.0 ± 8.2	33.0 ± 3.8

[a] LC 9018 (250 μg/mouse) was injected i.pl. into C57BL/6 mice (10 mice/group) on day 0
[b] Cytotoxic activity was measured by the ^{51}Cr release assay described in *Materials and methods*
[c] Effector/target ratio
[d] Mean ± SD of triplicate cultures

lung macrophages from mice injected with LC 9018 i.pl. LC 9018 was injected i.pl. and lung macrophages were collected on days 1, 3, 5, and 7. The cytolytic activity of the lung macrophages against 3LL was augmented on day 1 and the activity was maintained for 7 days (Table 6). These results suggest that the lung macrophages from mice injected with LC 9018 i.pl. played a key role in preventing lung metastasis.

Discussion

The present study demonstrated that i.pl. administration of LC 9018 into 3LL-bearing mice was effective in inhibiting pulmonary metastasis (Table 1) and that the combination of i.pl. and i.l. or i.v. administration was also effective in preventing pulmonary metastasis (Tables 2 and 3). Moreover, the cytolytic activity of thoracic macrophages and NK activity in the pleural effusion were augmented (Tables 4 and 5). The effect of i.pl. administration of LC 9018 on the induction of TEC was to increase the number of TEC (Fig. 1). The population of the TEC was mainly PMNs in the early stage and macrophages were dominant in the late stage. Furthermore, the cytolytic activity of the lung macrophages was enhanced by i.pl. administration of LC 9018 (Table 6).

Table 6. Cytolytic activity of lung macrophages from mice injected with LC 9018 i.pl. against 3LL

Days after LC 9018 injection[a]	Cytotoxicity[b]	
	50 : 1[c]	25 : 1
0 (control)	2.6 ± 3.8[d]	−0.9 ± 3.2
1	21.2 ± 0.6	15.2 ± 1.8
3	32.2 ± 10.1	20.8 ± 4.0
5	47.5 ± 1.8	25.8 ± 4.4
7	20.9 ± 1.8	16.7 ± 4.0

[a] LC 9018 (250 μg/mouse) was injected i.pl. into C57BL/6 mice (10 mice/group) on day 0
[b] Cytotoxic activity was measured by the ^{51}Cr release assay described in *Materials and methods*
[c] Effector/target ratio
[d] Mean ± SD of triplicate cultures

Several investiagors have reported that i.pl. treatment with BCG-CWS, N-CWS, or OK432 is effective for lung cancer patients with malignant pleurisy [15, 16, 18]. In animal experiments, it has been reported that i.pl. administration of BCG-CWS or N-CWS prolonged the survival of C57BL/6 mice bearing squamous cell carcinoma which was induced by the repeated i.pl. injections of benzo(a)pyrene with charcoal powder [17]. On the other hand, it has been reported that i.pl. treatment with BCG-CWS or N-CWS diminished the pleural effusion of lung cancer patients with malignant pleurisy in clinical trials. We also observed that i.pl. administration of LC 9018 completely eliminated the pleural effusion in BALB/c mice which were inoculated with Meth A tumor i.pl. Our present study indicated that i.pl. administration of LC 9018 augmented the macrophage function and NK activity in the pleural effusion. These findings indicate that NK cells in the pleural effusion play an important role in the elimination of the pleural fluid including the malignant cells.

We observed that i.pl. administration of LC 9018 inhibited lung metastasis in 3LL-bearing C57BL/6 mice. Although the mechanisms responsible for the inhibition of pulmonary metastasis were not clarified, we considered that the activated thoracic macrophages induced by i.pl. injected LC 9018 attacked the surface nodules of the lungs and that TEC induced by i.pl. injected LC 9018 released some mediators such as interleukin-1 (IL-1) or cytotoxic factors (CTF), and the mediators activated the lung macrophages, resulting in the inhibition of pulmonary metastasis. It has been reported that peritoneal macrophages of Kupffer cells activated by treatment with LC 9018 produce a higher level of CTF [5]. It was observed that peritoneal macrophages induced with LC 9018 produced a higher level of IL-1 than the untreated control macrophages (unpublished data). Thus, we considered that mediators released from macrophagees play a key role in the cytostatic activity of lung macrophages of C57BL/6 mice.

In conclusion, the present data show that i.pl. administration of LC 9018 inhibits lung metastasis and i.l. or i.v. injection was also effective for the inhibition of lung metastasis. Furthermore, the combination of i.pl. and i.l. or i.v. injection of LC 9018 was also effective in the inhibition of lung metastasis. This inhibitory effect is thought to be due to the activation of thoracic macrophages, NK cells, or lung macrophages. While the mechanisms by which i.pl. LC 9018 inhibits lung metastasis in experimental systems is not yet known, i.pl. LC 9018 therapy is expected to have a very beneficial effect in lung cancer patients in clinical trials. In fact, i.pl. LC 9018 therapy was given in a phase II study to patients with malignant pleurisy and great benefit was observed. Further investigations will be required to reveal the detailed mechnisms by cancer metastasis is prevented by LC 9018.

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